REMARKS

The Sequence Listing submitted herewith corrects errors and omissions in the Sequence Listing submitted for the parent application on August 17, 2001, USSN 08/843,572, now US Patent No. 6,388,172, of which the instant application is a divisional application.

It is brought to the attention of the Examiner that SEQ ID NO:3, referred to on page 12, lines 1 and 2 as the "sequence of clone 25" is present in the Sequence Listing submitted for the parent application. A copy of the Sequence Listing and the accompanying Amendment to enter into the Specification of the parent application is enclosed for the convenience of the Examiner. This sequence was, therefore, included in the Sequence Listing of the instant application without introducing new matter.

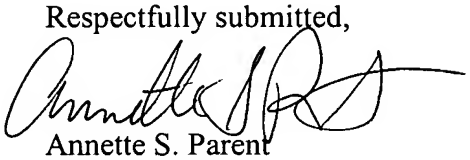
Applicants request entry of this amendment in adherence with 37 C.F.R. §§1.821 to 1.825. This amendment is accompanied by a floppy disk containing the above named sequences, SEQ ID NOS:1-27, in computer readable form, and a paper copy of the sequence information which has been printed from the floppy disk.

The information contained in the computer readable disk was prepared through the use of the software program "PatentIn" and is identical to that of the paper copy. This amendment contains no new matter.

Attached hereto is a marked-up version of the changes made to the Specification by the current Amendment. The attached pages are captioned "**VERSION WITH MARKINGS TO SHOW CHANGES MADE.**"

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Paragraph beginning at line 10 of page 5 has been amended as follows:

Figure 1 gives sequences of the A2 fragment of the *Lhcb1*3* promoter (SEQ ID NO:14) and of DNA fragments used in EMSA analysis along with indications of nucleotide modifications that reduce CCA1 binding; in the probe sequences (WT1, m1, m2, m3, m4, and WT2; SEQ ID NOS:15-20, respectively) dashes indicate those nucleotides that are identical to the A2 probe while dots denote gaps introduced to optimize the alignment of conserved sequence elements;

Paragraph beginning at line 15 of page 5 has been amended as follows:

Figure 2 shows the complete nucleic acid sequence of *CCA1* (SEQ ID NO:1), the genomic clone corresponding to the CCA1 cDNA along with the deduced amino acid sequence (SEQ ID NO:2) of the coding portions of the gene;

Paragraph beginning at line 1 of page 6 has been amended as follows:

Figure 3 shows the predicted amino acid sequence of CCA1 from amino acid residue 24 to 75 (SEQ ID NO:21) compared to the repeat sequences of various Myb proteins (SEQ ID NOS:22-27) from animals, plants, and yeast;

Paragraph beginning at line 15 of page 6 has been amended as follows:

Figure 7 shows on the left the results of EMSA with the A2 fragment and the amount of proteins and poly (dI-dC) shown above each lane; F, free probe; B, CA-1 protein-DNA complex; B1 and B2, CCA1 protein-DNA complexes; on the right is shown the sequencing gel of the cleaved DNA recovered from the phenanthroline-copper reaction with S lanes representing the G+A chemical sequencing reaction and with the actual sequence of the protected region (SEQ ID NO:10) spelled out;-

Paragraph beginning at line 5 of page 15 has been amended as follows:

Genomic DNA isolation and DNA gel blotting were performed as described by Brusslan et al. (1993). Membranes were hybridized with ^{32}P-labeled CCA1 cDNA fragments under high stringency conditions (final washes were at 65°C in 0.1% SSC, [1X SSC is 0.15 M NaCl, 0.015 M sodium citrate] 0.1% SDS) and then stripped and reprobed under low-stringency conditions (hybridization at 32°C in buffer containing 50% formamide, 0.25 M $NaHPO_4$, pH 7.2, 0.75 M NaCl, 7% [w/v] SDS, and 1 mM EDTA and final washes at 45°C in 2X SSC, 0.1% SDS). Total RNA was extracted from *Arabidopsis* seedlings as described by Brusslan and Tobin (1992). Total RNA was separated on a 1% agarose gel containing formaldehyde and blotted onto ZetaProbe membrane (Bio-Rad, Richmond, CA) following the manufacturer's instructions. RNA probes were synthesized by in vitro transcription using linearized plasmid DNA. *CCA1* RNA probe was synthesized from CCA1 clone 24. To make the *ubql0* RNA probe, a fragment of the 3' untranslated region of the *ubql0* gene (Callis, J., Carpenter, T., Sun, S.W., and Vierstra, R.D., *Genetics* 139:921-39 (1995)) was amplified by polymerase chain reaction (PCR) using the primers:

5'-CTGTTATGCTTAAGAAGTTCAATGT-3' (SEQ ID NO:4)
and 5'-CCACCCTCGAGTAGAACACTTATTCAT-3' (SEQ ID NO:5).

The amplified fragment was digested with HindIII and XhoI and cloned into pGEM-llZf(-). This plasmid DNA was digested with HindIII and used as template for synthesis of *ubql0* RNA probe. The *Lhcbl*3* RNA probe was made as described by Brusslan and Tobin (1992). The membrane blot was hybridized overnight with the RNA probes in buffer containing 50% formamide, 0.3 M NaCl, 0.05 M $NaHP0_4$, pH 6.5, 1 mM EDTA, 1% SDS, 0.1% Ficoll (type 400), 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.5 mg/mL yeast tRNA and 0.5 mg/mL herring sperm DNA. Hybridization of *Lhcbl*3 ubql0* and *CCA1* probes was performed at 55°C, 52°C and 58°C, respectively. Final washes were performed at 65°C in 0.1X SSC, 0.1% SDS. After hybridization with *Lhcbl*3* and *ubq10* probes, the blot was stripped by boiling in 0.1X SSC, 0.1% SDS, then hybridized with the *CCA1* probe. The blots were imaged and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The measurement of the signal for each probe was adjusted for the uridine content of the probe and the exposure time, and the *Lhcbl*3* and *CCA1* signals were normalized to the *ubq10* signal.

Paragraph beginning at line 12 of page 19 has been amended as follows:

The constructs diagrammed in Figure 5 were made by cloning the CCA1 cDNA fragments into pGEX-3X (Pharmacia) using polymerase chain reaction (PCR)-aided cloning with the following 5' primers:
5'-GGCCGGGATCCAATTCGTCGACCCACGCG-3' (SEQ ID NO:6) for pXCA-21, pXCA-24, pXCA-25 and 5'-TAAAGGGATCCATATGGGTCAAGCGCTAG-3' (SEQ ID NO:7) for pXCA-23. A 3' primer (5'-ATAGAATTCTCGAGCTTATGCATGCGG-3'; SEQ ID NO:8) was used for pXCA-21, pXCA-24, pXCA-25 and pXCA-23. The appropriate plasmid DNA (0.5 μg) was amplified for 10 cycles and the PCR products were digested with EcoRI and BamHI. The cDNAs of clones 21, 24, and 25 and the 483 to 2254-nucleotide region were cloned into -pGEX-3X yielded pXCA-21, pXCA-24, pXCA-25 and pXCA-23, respectively. Sequencing of the junction region between the

glutathione *S*-transferase (GST) gene and cDNA confirmed the construction of a translational fusion in pXCA-24 and pXCA-23.

Paragraph beginning at line 14 of page 21 has been amended as follows:

To compare the binding characteristics of the CCA1 protein and CA-1 activity from the plants, we carried out footprint analyses and binding competition experiments using the A2 fragment of *Lhcbl*3* promoter as a probe. The results of 1,10-phenanthroline-copper footprinting are shown in Figure 7. At left is the EMSA that was performed to resolve the free probe and the DNA-protein complexes. Cleaved DNA was recovered from each band after treatment of the gel with phenanthroline-copper and resolved on the sequencing gel shown at right. With increasing amounts of the CCA1 protein purified from *E. coli*, complexes (B-1 and B2) of different mobilities could be observed. The nucleotides protected from cleavage in each of the complexes can be seen on the sequencing gel on the right. In complex B1, the -92 to -105 region was protected, and in complex B2, regions from -92 to -105 and from -111 to -122 were protected. This result suggests that the two complexes of different mobilities are a result of the presence of two separate binding sites on this fragment, and that the -92 to -105 region is the higher affinity binding site for CCA1. A nearly perfect repeated sequence of AAA$^{A}/_{C}$AATCTA (SEQ ID NO:9) occurs in each of these footprinted regions.

Paragraph beginning at line 15 of page 22 has been amended as follows:

Figure 8 shows ~~show~~ the results of methylation interference and depurination interference experiments performed with the CCA1 protein. The figure shows the interfering nucleotides on sequencing gels, and their position on the A2 fragment of the promoter is shown in Figure 1, along with the results of footprinting experiments. Interference with the protein-DNA binding by the modification of a base residue is manifested by increased intensity in the lane with the free DNA fraction and reduced

intensity in the lane of protein-bound DNA compared to the lane of control DNA that was not incubated with protein. Both methylation and depurination interference assays identified the same nucleotides, and showed that nucleotides within both nearly perfect repeats (AA^{A}/$_{C}$AATCTA; SEQ ID NO:9) interact directly with the CCA1 protein. In Figure 1 thick and thin lines 12 and 14 show the regions protected by CCA1 and CA-1, respectively, in the footprint assay. Asterisks in the figure indicate nucleotides that interfere with CCA1-DNA binding when methylated; boldface indicates the nucleotides that interfere with binding when depurinated.

Paragraph beginning at line 10 of page 23 has been amended as follows:

Figure 1 also summarizes the results of the phenanthroline-copper footprinting. We used unlabeled competitor DNAs in the EMSA to compare binding specificities of the CCA1 protein produced in *E. coli* and the CA-1 activity from the plant extracts. The wild-type and mutant promoter fragments used as competitors are shown at the bottom of Figure 1. A representative result of such experiments is shown in Figure 9a for CCA1 and Figure 9b for CA-1. The binding of the *E. coli*-produced CCA1 protein to the probe was efficiently competed for by either a fragment of the A2 probe that contained the repeated sequence or by a promoter fragment (WT2) of another closely related *Lhcb* gene (*Lhcbl*l*, originally called AB165; Leutwiler, L.S., Meyerowitz, E.M., and Tobin, E.M., *Nucl. Acids Res.* 14:4051-64 (1986)) that contains one copy of this sequence (AAAAATCT; SEQ ID NO:11). The m3 fragment, which had altered nucleotides in the downstream repeat region, was a less effective competitor than was the wild-type (WT1); ml, m2, and m4 fragments, which had alterations in both repeats, showed the least competition.

Paragraph beginning at line 4 of page 24 has been amended as follows:

When plant extracts were used, all the fragments showed some degree of competition, which is likely in part to be the result of low amounts of the CA-1 protein in plant extracts. The results are not directly comparable to those with the purified CCA1 protein because the absolute amounts of the specific binding proteins are not known. Nonetheless, it can be seen that the m2 fragment served as a better competitor for CA-1 than did the ml fragment, whereas the opposite was found with CCA1. Even more striking are the contrasting results with the m4 competitor DNA. This fragment, in which the C residues of both TCT motifs in the two repeats were changed, was even more effective than was the wild type in competing for the CA-1 activity, whereas it was not a particularly good competitor for CCA1. Thus, although both activities interact with the AAAAATCT (SEQ ID NO:11) sequence, there are differences in the importance of individual nucleotides in this sequence for the binding of CA-1 and CCA1.

Paragraph beginning at line16 of page 24 has been amended as follows:

The CCA1 protein interacted with two closely spaced binding sites with nearly perfect 10-bp repeated sequences (AAA$^{A/C}$AATCTA; SEQ ID NO:9) in the *Lhcbl*3* promoter. Previous results (Sun et al., 1993) and the results of the phenanthroline-copper footprinting (Figure 7) show that the CA-1 activity could protect the same nucleic acid sequence as CCA1. There are, however, some differences in the relative importance of specific nucleotides for the binding of the two activities. The binding of CCA1 was more affected by alteration in the TCT sequence than by alterations in the AAAAA, whereas the opposite was observed with the plant extract activity (cf. m3 and m4, Figure 9). It is possible that the differences observed are due to differences in modifications of the protein in *E. coli* and plants or that the CA-1 activity in the plant extracts might be associated with an additional protein or proteins which alter the binding characteristics. It is also possible that CA-1 and CCA1 are actually the products of two different genes, or

the result of alternative splicing, in which case they may compete for the same binding sites.

Paragraph beginning at line17 of page 25 has been amended as follows:

Transient expression assay in onion epidermal cells tested whether the product of the CCA1 gene was localized to nuclei, as would be expected for a transcription factor. The uidA gene, which encodes, β-glucuronidase (GUS), was fused in frame to the coding sequence of *CCA1* so that GUS activity could be used to localize the compartmentation of the CCA1 protein. An XbaI site and a BamHI site were introduced into CCA1 ~~CCA1~~ by PCR amplification of cDNA clone 25 using the 5' primer-(5'-GAAGTTGTCTAGAGGAGCTAAGTG-3'; SEQ ID NO:12) and 3' primer (5'-ATGTGGATCCTTGAGTTTCCAACCGC-3'; SEQ ID NO:13) (mismatches are underlined). The resulting PCR product was digested with XbaI and BamHI and inserted in pBI221 (Clontech, Palo Alto, CA), yielding p35S-CCA1-GUS. This construct contains *CCA1*-coding ~~*CCA1* coding~~ sequence as a 1828-bp XbaI-BamHI fragment inserted between the cauliflower mosaic virus 35S promoter and the uidA gene. pMF::GUS and pMF::B::GUS were obtained from N. Raikhel (Michigan State University, East Lansing, MI); construction of these plasmids is described in Varagona et al. (1992). This transient assay should result in the expression of GUS activity in individual cells into which the DNA is effectively introduced. When a CaMV 35S::uidA construct (pMF::GUS) was used in this assay, GUS activity was detected throughout the cytoplasm. When a construct (pMF::B::GUS) with the *opaque2* gene, which encodes a well characterized transcription factor from maize, fused to the *uidA* gene was used, GUS activity was detected specifically in nuclei. Similarly, specific nuclear localization was found for the CCA1-GUS (p35S-CCA1-GUS) fusion protein. These results show that the CCA1 protein is targeted to nuclei and are consistent with the function of the CCA1 protein as a transcription factor.